targeted application of transgenes to a specific species, such as the use of growth hormone constructs in fish (Chapter 15) or the attempts to transfer two genes in a biochemical pathway to alter the intermediary metabolism of mammals (Chapter 12). In each chapter, we hope to convey to the reader a better understanding of the possibilities and limitations of the current state of our art and the excitement of the participants at this meeting as we try to apply transgenic technology to help improve the animals used in agriculture.

Finally, as editors and organizers, we need to thank those people other than the speakers and authors who gave freely of their time to assist us in bringing together first the conference and now this volume. A brief list includes Robert Devlin, James Petitte, Carl Pinkert, Caird Rexroad Jr and George Seidel, who helped us to plan the scientific programme and to identify speakers. We also extend our thanks to a large number of anonymous referees who provided the peer review for each manuscript and in so doing provided valuable comments to both the authors and to us. The conference, and thus this volume, would not have been possible without financial support from the University of California, Davis, grants from the University of California Systemwide Biotechnology Research and Education Program and the USDA-CSREES, and contributions from the following corporate sponsors: Bayer Pharmaceuticals, Wyeth-Ayerst Laboratories, Perkin Elmer ABI, Biogenics, Metamorphix, PPL Therapeutics, Pharming B.V., Genzyme Transgenic Corporation and BioTime Corporation. This book would never have been finished without Randy Cook's unflagging assistance in helping to pull it all together. Finally, we would like to acknowledge Mr Tim Hardwick from CABI *Publishing*, both for his encouragement to undertake the preparation of this volume and his patience as time went by.

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Transgenic Farm Animals

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The last quarter-of-a-century has witnessed a rapid advance in the application of genetic engineering techniques to increasingly complex organisms, from bacteria and yeasts to mammalian species. In 1985, the first report on the production of genetically engineered farm animals described transgenic rabbits, sheep and pigs. Since that time, in addition to models for a number of mammalian species, transgenic fish and bird models have also been developed. Transgenic animals have provided us with a means of analysing developmental and regulatory mechanisms *in vivo*. Areas of research focusing on production characteristics have targeted growth and development, disease resistance, reproduction, lactational performance, feed efficiency, immune responsiveness and fibre production. Additionally, novel biomedical applications have forged ahead, using transgenic farm animals as research models and as bioreactors to produce biologically important proteins, tissues and organs for a host of specific applications. Today, transgenic animals embody one of the most potent and exciting research tools in the agricultural and biological sciences. These genetically engineered animals can be custom tailored to address specific scientific questions that were previously beyond our reach. Transgenic animal technology is of particular relevance in the rapid genetic modification of farm animal species, especially when one considers that selective breeding, which can be used to direct the modification of a specific phenotype, cannot be used to engineer a specific genetic trait in a directed fashion. As we enter the 21st century, novel methods to enhance the efficiency of transgenic animal production and to increase the utility of transgenic animal models in agriculture and society continue to evolve.

Introduction

The scientific breakthroughs that have enabled the current successes in the genetic engineering of animals occurred over the past century beginning

with the first attempts to culture and transfer embryos in the late 1800s (Table 1.1). While recent progress seems extremely rapid, it is still difficult to believe that, following the first published report of a microinjection method (Lin, 1966), 15 years passed before the first transgenic mice were created by Gordon *et al.* (1980). The first technological shift toward transgenic mouse production occurred in 1977, when Gurdon transferred mRNA and DNA into *Xenopus* embryos and observed that the transferred nucleic acids could function in an appropriate manner. Then, in 1980, Brinster and his colleagues reported on similar studies in the mouse. They demonstrated that an appropriate translational product was produced following transfer of a specific mRNA into mouse embryos. Sequentially, these studies laid the groundwork for the development of the first 'gain-of-function' transgenic mouse models.

From late 1980 through 1981, six research groups reported success in gene transfer and the development of transgenic mice. To describe animals carrying new genes (integrating foreign DNA into their genome), Gordon and Ruddle (1981) coined the term 'transgenic'. This definition has since been extended to include animals that result from the molecular manipulation of endogenous genomic DNA, including all techniques from DNA microinjection to embryonic stem (ES) cell transfer and 'knockout' mouse production.

Table 1.1. Transgenic animal milestones.

0000	genetic selection to improve animal productivity
1880	mammalian embryo cultivation attempted
1891	first successful embryo transfer
Early 1900s	*in vitro* embryo culture develops
1961	mouse embryo aggregation to produce chimeras
1966	zygote microinjection technology established
1973	foreign genes function after cell transfection
1974	development of teratocarcinoma cell transfer
1977	mRNA and DNA transferred into *Xenopus* eggs
1980	mRNA transferred into mammalian embryos
1980–1981	transgenic mice first documented
1981	transfer of ES cells derived from mouse embryos
1982	transgenic mice demonstrate an enhanced growth (GH) phenotype
1983	tissue-specific gene expression in transgenic mice
1985	transgenic domestic animals produced
1987	chimeric 'knock-out' mice described
1989	targeted DNA integration and germline chimeric mice
1993	germline chimeric mice produced using co-culture
1994	spermatogonia cell transplantation
1997	nuclear transfer using ES and adult cell nuclei in sheep
1998	nuclear transfer using ES cells to derive transgenic sheep
2000	????

Since the early 1980s, the production of transgenic mice by microinjection of DNA into the pronucleus of zygotes has been the most productive and widely used technique. Using transgenic technology in the mouse, such as antisense RNA encoding transgenes, it is now possible to add a new gene to the genome, increase the level of expression or change the tissue specificity of expression of a gene, and decrease the level of synthesis of a specific protein (see Sokol and Murray, 1996). Removal or alteration of an existing gene via homologous recombination required the use of ES cells and was limited to the mouse until the advent of nuclear transfer cloning procedures (Wilmut *et al.*, 1997; see also Chapter 5, this volume).

This review notwithstanding, there are now literally hundreds of excellent reviews that detail the production and utility of transgenic animals. (A number of reviews and texts are cited in the references in addition to a journal, *Transgenic Research*, which is dedicated to this field.) Yet, the most influential experimentation to impact on transgenic farm animal research was the work of Palmiter and Brinster in the early 1980s. Their studies related to growth, performance and the dramatic phenotype of mice transgenic for growth hormone (GH), influenced animal agriculture in dramatic fashion. In these pioneering studies 'Super Mice', which grew 100% larger than normal or littermate mice, were produced by redirecting GH production to the mouse's liver, using a liver-specific metallothionein promoter fused to a GH structural gene (e.g. Palmiter *et al.*, 1982).

During the past 15 years, transgenic technology has been extended to a variety of animal species beyond the mouse, including rats, rabbits, swine, ruminants (sheep, goats and cattle), poultry and fish (Table 1.2). With advances in the understanding of promoter-enhancer elements and transcription-regulatory proteins involved in the control of gene expression, the technology continues to evolve using different model systems (Box 1.1). In the systems explored to date, gene transfer technology is a proven asset in science as a means of dissecting gene regulation and expression *in vivo*.

Table 1.2. Genetically engineered vertebrate species.

Mammals	Birds	Fish
Mice	Chickens	Salmon
Rats	Japanese quail	Trout
Rabbits		Tilapia
Cattle		Carp
Pigs		Catfish
Sheep		Medaka
Goats		Zebrafish
		Loach
		Goldfish
		Pike

Box 1.1. Application and use of transgenic animal models.

Transgenic animals have provided models in agricultural, biomedical, biotechnological and veterinary disciplines in the study of gene expression and developmental biology, as well as for modelling:

- Increased efficiency of animal production.
- Genetic bases of animal and human diseases (leading to the design and testing of strategies for therapy).
- Gene therapy.
- Disease resistance in animals and in humans.
- Drug and product efficacy testing/screening.
- Novel or improved product development, 'molecular farming', ultimately targeting products or productivity of domestic animals. Models range from enhancing production traits of interest to 'foreign' protein production and human organ replacement (xenotransplantation).

As such, the primary questions that are addressed concern the roles of individual genes in development or in particular developmental pathways. With this caveat, considerations include the ramifications of gene activity, from intracellular to inter- and extracellular events within a given tissue or cell-type milieu.

Gene transfer has been used to produce both random and targeted insertion of discrete DNA fragments into the mouse genome. For targeted insertions, where the integration of foreign genes is based on a recombinational gene insertion with a specific homology to cellular sequences (termed homologous recombination), the efficiency at which DNA microinjection is effective is extremely low (Brinster *et al.*, 1989). In contrast, the use of ES cell transfer into mouse embryos has been quite effective in allowing an investigator to preselect a specific genetic modification, via homologous recombination, at a precise chromosomal position. This preselection has led to the production of mice: (i) incorporating a novel foreign gene in their genome, (ii) carrying a modified endogenous gene, or (iii) lacking a specific endogenous gene following gene deletion or 'knock-out' procedures (see Capecchi, 1989; Brinster, 1993).

Isolation and propagation strategies for ES cells in domestic species have proven elusive, with much of the effort now being directed towards the isolation of primordial germ (PG) cells. Techniques such as nuclear transfer might use donor nuclei from various sources (e.g. ES cell, embryonic cell lines, PG cells or spermatogonia) to produce offspring. The utility of ES cells or related methodologies to provide efficient and targeted *in vivo* genetic manipulations offer the prospects of profoundly useful animal models for biomedical, biological and agricultural applications. The road to such success has been most challenging, but recent developments in this field are extremely encouraging.

Production of Transgenic Domestic Animals

The success of transgenic mouse experiments led a number of research groups to study the transfer of similar gene constructs into the germline of domestic animal species. With one exception, these efforts have been directed primarily toward either of two general goals: (i) improving the productivity traits of domestic food animal species, or (ii) developing transgenic lines for use as 'bioreactors'; i.e. as producers of recoverable quantities of medically or biologically important proteins. These studies revealed basic biological mechanisms as well as a need for precise regulation of gene expression. Since 1985, transgenic farm animals harbouring growth-related gene constructs have been created, although ideal growth phenotypes were not achieved because of an inability to coordinately regulate either gene expression or the ensuing cascade of endocrine events (see Pursel *et al.*, 1989; Pursel and Rexroad, 1993; Pinkert *et al.*, 1997).

Presently, DNA microinjection and now nuclear transfer (Schnieke *et al.*, 1998) are the only methods used to produce transgenic livestock successfully. Although involved and at times quite tedious, the steps in the development of transgenic models are relatively straightforward. For either DNA microinjection or nuclear transfer, once a specific fusion gene has been cloned and characterized, sufficient quantities are isolated, purified and tested in cell culture if possible. Once the appropriate gene construct has been identified, the fragment is linearized, purified and readied for preliminary mammalian gene transfer experiments. In contrast with nuclear transfer studies, DNA microinjection experiments are first performed in the mouse. While the transgenic mouse model will not always identify likely phenotypic expression patterns in domestic animals, we have not observed a single construct that would function in a pig when there was no evidence of transgene expression in mice. Therefore, preliminary experimentation in mice has been a crucial component of any gene transfer experiment in domestic animals.

With the exception of recently reported nuclear transfer experiments in sheep and cattle, there has been little change in the methods used to produce transgenic mammals, birds and fish over the last few years. For the sake of brevity, further discussion in this paper will be centred around the production of transgenic livestock in order to illustrate some points concerned with the production, utilization and limitations of transgenic animals in general. In practice, except for the nuclear transfer reports by Wilmut in sheep (Schnieke *et al.*, 1998) and Robl in cattle (Cibelli *et al.*, 1998), all other transgenic farm animals to date have been produced by pronuclear microinjection and in all cases the efficiency of producing transgenic animals is low (Table 1.3; also see Wall *et al.*, 1992). While nuclear transfer might be considered inefficient in its current form, we anticipate major strides in enhancing experimental protocols within the next few years, comparable perhaps with the early advances in DNA

Table 1.3. Efficiency of producing transgenic farm animals (percentage of transferred microinjected zygotes).

Species	Born	Transgenic
Pig	9.9	0.91
Sheep	10.6	0.88
Goat	14.3	0.99
Cattle*	16.2	0.79
Mice	15.0	≤3.5

* Based on transfer of morulae/blastocysts.
Modified from Pursel and Rexroad (1993).

microinjection technology. The added possibility of gene targeting through nuclear transplantation opens up a host of applications, particularly with regard to the use of transgenic animals to produce human pharmaceuticals (see Pinkert, 1997).

The current state of the art for the production of transgenic farm animals is still relatively unchanged from what it was 13 years ago; however, there are a host of procedures in development that may very well change 'state-of-the-art' technology very shortly. The only major technological advance since the initial production of transgenic farm animals has been the development of methods for the *in vitro* maturation of oocytes (IVM), *in vitro* fertilization (IVF) and subsequent culture of injected embryos prior to transfer to recipient females at some point up to, and including, the early blastocyst stage (Gordon and Lu, 1990). IVM and IVF have made the production of transgenic cattle economically feasible, even though the overall efficiency is low. Considerable effort has been expended towards establishing ES cells for cattle, sheep, chickens and pigs, but to date without success. While the techniques currently used to produce transgenic animals are inefficient, a variety of species can be, and are, routinely genetically engineered. This suggests that 'new' types of transgenic farm animals will continue to be produced for some time.

The major limiting factor in the production of transgenic mammals is the rate at which the microinjected DNA is integrated into the recipient genome (Wall *et al.*, 1992). However, to date, there has been virtually no research done to ascertain the mechanism(s) responsible for integration. Once the mechanism of integration is known, it may be possible to develop techniques to enhance the rate of transgene incorporation and thus gain significant efficiencies in the overall rate at which transgenic mammals can be produced.

Using DNA microinjection, the types of genes and regulatory sequences introduced into livestock species become important considerations. Pursel and Rexroad (1993) provided a comprehensive list of gene constructs used in the production of transgenic cattle, goats, pigs and sheep that has not changed significantly over the last 4 years. Table 1.4 summarizes their data

Table 1.4. Number of genes transferred into livestock.

Species	Growth factors	Milk genes	Total
Pig	15	2	23
Sheep	5	2	11
Goat	—	2	2
Cattle	4	1	6

with respect to the total number of genes transferred into each species and the two principal functional types of coding sequences. As can be seen, the types of transgenes used fall into two main types: those encoding growth factors and those encoding proteins for expression in the mammary gland.

The work with growth factors was carried out in an attempt to alter the efficiency of meat production and alter the partitioning of nutrient resources towards increased lean production; i.e. these projects were intended to alter animals for use in production agriculture. To date, these attempts have failed to result in the production of genetically superior livestock (sheep and pigs) due to a variety of undesirable side effects in these animals, although in general the transgenic animals have been more feed efficient and leaner (Pursel *et al.*, 1989; Nancarrow *et al.*, 1991). In addition to the work with livestock transgenic for growth factor, considerable effort has been directed towards increasing the efficiency of wool growth in Australian sheep by insertion of the two bacterial or yeast genes required for sheep to synthesize *de novo* the sulphur amino acid cysteine (see Rogers, 1990; Ward and Nancarrow, 1991; Chapter 12, this volume).

Work on the directed expression of new proteins with pharmaceutical value to the mammary gland of cattle, goats, pigs and sheep has been more successful. A number of pharmaceutically important proteins have been expressed in the mammary gland, with human α_1-antitrypsin being expressed in sheep milk (Archibald *et al.*, 1990; for review see Maga and Murray, 1995; Pinkert, 1997) at levels high enough for consideration for commercial extraction. While pharmaceutical-producing farm animals will continue to be developed, they will not have a direct effect on agriculture and, as there is high value in the protein being produced, it would not even be necessary for these animals to ever enter the human food chain. Thus, the value of this work to agriculture is in the knowledge gained concerning the control of mammary gland gene expression and the potential development of new techniques to increase the efficiency of producing transgenic farm animals.

Yet, the major scientific limitations to the wide-scale application of transgenic technology to improve farm animals basically have not changed since 1986 (Ward *et al.*, 1986). Those limitations include:

1. Lack of knowledge concerning the genetic basis of factors limiting production traits.

2. Identification of tissue- and developmentally specific regulatory sequences for use in developing gene constructs, expression vectors and in gene targeting.
3. Establishment of novel methods to increase the efficiency of transgenic animal production.

The production of transgenic farm animals is not undertaken lightly due to the high costs associated with obtaining and maintaining these animals. Thus it is prudent to confirm transgene expression in mice before it becomes cost effective to initiate DNA microinjection experiments in other species. In mouse experiments, less than 2 months is required from the time the purified construct is ready for microinjection until the weaning of founder pups. In contrast, for pig experiments, 1 month to a year is required for a sufficient number of DNA injections and recipient transfers to ensure the likelihood of success. Experimental efficiencies coupled with a long generational interval (i.e. 114 day gestation period, 21–28 day lactation and onset of puberty at 6–9 months of age) reflect the efforts necessary to identify and characterize transgenic pigs and illustrate the extended timelines associated with the production of any transgenic livestock model. In addition, the time-frame from birth of a founder transgenic animal to the establishment of lines can be 1–2 years for pigs, sheep and goats to 4–5 years for cattle (while also dependent on the sex of founders). Hence, there is an obvious advantage to characterizing transgenic mouse models to expedite what will ultimately be a lengthy undertaking.

More recently, protocols were developed to permit removal of individual blastomeres from microinjected pre-implantation embryos maintained in culture prior to transfer to recipient females followed by PCR analysis of DNA purified from individual blastomeres to identify those embryos that bear the transgene of interest. Use of such methods has the potential to greatly increase the efficiency associated with production of transgenic farm animals and to thereby significantly reduce the associated costs. However, to date the potential increase in efficiency due to the identification of embryos carrying the transgene prior to embryo transfer is offset by a loss of viability of the biopsied embryo and the occurrence of false negatives and false positives in the PCR analysis (e.g. Behboodi *et al.*, 1993; Horvat *et al.*, 1993).

Strain and Species Considerations

Transgenic techniques have been developed for a variety of vertebrate species in addition to the mouse (Table 1.2). However, the most informative system is encountered in the production of transgenic mice, simply because so much work has been done with this species. In mice, differences in reproductive productivity, behaviour, related husbandry requirements and

responses to various experimental procedures that affect overall production efficiency are well documented. Additionally, strain differences may have significant influences on modifying gene expression; e.g. gene expression and tumour formation in lines of transgenic mice harbouring human oncogenes (or with tumour suppressor genes 'knocked out') vary when these mice are backcrossed to different inbred or outbred strains (Harris *et al.*, 1988; Chisari *et al.*, 1989; Cho *et al.*, 1989; Donehower *et al.*, 1995).

DNA microinjection protocols developed in mice have been modified to accommodate production of other transgenic species. Differences between these species and mice in the embryo quality and physical response to microinjection, requirements for embryo culture, quantity of embryos needed for embryo transfer and pregnancy maintenance, as well as differences in general husbandry practices, are well documented.

To this point, we have not mentioned the production methods used to produce transgenic poultry and fish. In both instances, genetic selection is an exceedingly slow process. Since DNA microinjection into pronuclei of embryonic cells in poultry is not feasible, transfection methodologies using replication-competent and replication-compromised retroviruses has taken centre-stage (Shuman, 1991; Perry and Sang, 1993; Cioffi *et al.*, 1994). As described, methods have included transfection of genes into cells of embryonic blastoderm; insertion of genes using replication-competent retroviruses; the use of replication-defective retroviruses; and sperm-mediated gene transfer. While the latter method has come under critical dispute, the other methods have led to the development of experimental models.

In contrast with poultry studies, work with fish has moved ahead with far greater speed. The principal area of research has focused on growth performance, and initial transgenic GH fish models have demonstrated accelerated and beneficial phenotypes (Fletcher and Davies, 1991; Houdebine and Chourrout, 1991; Cioffi *et al.*, 1994). DNA microinjection methods have propelled the many studies reported and have been most effective due to the relative ease of working with fish embryos. Ideally, efforts at developing 'mass transfer' techniques (e.g. electroporation, sperm binding and lipofection-mediated transfer) would aid in commercializing transgenic fish for the aquaculture industry.

Stem Cells and Alternative Methods for Gene Transfer

The development of ES cell technologies emanated from efforts of the early cell biologists. Teratocarcinoma cell transfer and cell aggregation work in the 1970s evolved from the earlier characterization and studies of teratocarcinoma cells (Pierce, 1975; see also Brinster, 1993; Pinkert, 1997). This led to work with the '129' mouse strain and pluripotential teratocarcinoma cells, and then ultimately to the basis for work with embryonic carcinoma and stem cells in 1981 (Evans and Kaufman, 1981; Martin, 1981). By 1985,

purified mouse ES cells were characterized, and by 1987 homologous recombination, gene targeting and the production of chimeric 'knock-out' mice ushered in a new era of 'loss-of-function' mutants to accompany existing techniques (Thomas and Capecchi, 1987; see also Capecchi, 1989; Brinster, 1993). Then, in a relatively brief period, the ability to target DNA integration (as opposed to *random integration* of microinjected genes) and to produce germline-competent chimeric mice was demonstrated. Within a few more years experimental efficiency was enhanced by the development of co-culture techniques, where blastocyst injection was not the only route for ES cell transfer. With co-culture, host embryos could be cultured on a lawn of ES cells, with the ES cells preferentially being incorporated into the embryo proper. Yet, in all of these cases, techniques continuously improved in incremental steps. Thus, the recent successful 'cloning' of a sheep (Wilmut *et al.*, 1997) has captured the imagination of researchers around the world. This technological breakthrough should play a significant role in the development of new procedures for genetic engineering in a number of mammalian species. It should be noted that nuclear cloning, with nuclei obtained from either mammalian stem cells or differentiated 'adult' cells, is an especially important development in 'non-mouse' species. This is because, until the report by Schnieke *et al.* (1998), germline-competent transgenics had only been produced in mammalian species, other than mice, using DNA microinjection.

In contrast with progress in embryo manipulation, a completely different tack was taken with the advent of sperm-related transfer procedures. In 1989, sperm-mediated gene transfer was reported but hotly disputed when many laboratories around the world were unable to duplicate the procedures. Yet, by 1994, the sperm-mediated story generated interest that resulted in the development of spermatogonial cell transplantation procedures as a potentially feasible alternative for gene transfer experimentation (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994). With embryo- and sperm-related procedures leading the way, as we move into the 21st century, many of our existing procedures will continue to evolve and become more practicable (Box 1.2). However, whole-animal and somatic cell techniques (including liposome-mediated gene transfer, particle bombardment and jet injection), coupled with novel vectors and vector design, will continue in their evolution and in enhancing our gene-transfer capabilities.

Gene Transfer and Gene Regulation

The various strategies for producing genetically engineered animals extend from the mechanistic (e.g. DNA microinjection, ES cell- or retroviral-mediated transfer) to the requisite gene cloning and modelling techniques. However, our understanding of promoter-enhancer sequences and external

Box 1.2. Gene transfer methodologies.

Mouse modelling techniques have evolved from procedures for non-specific (whole genome) transfer, as in aggregation and teratocarcinoma studies, to the transfer of discrete genes and the modification of endogenous genes.

- Blastomere/embryo aggregation.
- Teratocarcinoma cell transfer.
- Retroviral infection.
- Microinjection.
- Electrofusion.
- Nuclear transplantation.
- Embryonic stem (ES) cell transfer.
- Spermatozoa- and spermatogonial cell-mediated transfer.
- Particle bombardment and jet injection.

transcription-regulatory proteins involved in the control of gene expression continues to advance using different model systems. In the systems explored to date, gene transfer technology is a proven asset in science as a means of dissecting gene regulation and expression *in vivo*. However, the primary type of question that is addressed in these systems still concerns the particular role of a single gene in development or in a given developmental pathway.

The three major factors that influence gene expression in all animals, but are particularly relevant to transgenic animals, include: *cis*-acting elements, *trans*-acting factors and the specific gene location (insertion site) within the genome. *Cis*-acting elements determine the state of chromosomal accessibility and consequently the tissue distribution and developmental timing of gene expression. *Cis*-acting elements act in proximity to a given gene and include both promoters and enhancers. Promoters are location-dependent regions of DNA involved in the binding of RNA polymerase to initiate gene transcription. Enhancers are location-independent sequences (they function in either orientation, upstream or downstream of a promoter), and increase the utilization of promoters. In contrast, *trans*-acting factors interact with genes in open domains and stimulate transcription. Normally, gene function is influenced by both *cis*-acting elements and *trans*-acting factors. For transferred genes, the *cis*- and *trans*-activators work in conjunction with the gene integration/insertion event. The chromosomal environment is a major factor that influences gene expression (i.e. the insertion site may alter expression of an endogenous gene), as seen when a gene fails to function (express) in one or more lines of transgenic animals, while it is active in other lines. Using genes that code for reporter proteins (e.g. GH or *lac*Z constructs), analysis of transgenic animals has revealed the importance of these three factors in determining developmental timing, efficiency and tissue distribution of gene expression. Additionally, transgenic animals have proven quite useful in unravelling *in vivo* artefacts of other non-transgenic model systems and techniques. Interestingly, regulation of

specific genes in one species does not always correspond to species-specific homologues or the regulation seen in other species.

Traits Affecting Domestic Animal Productivity

Interest in modifying traits that determine the productivity of domestic animals was greatly stimulated by early experiments in which body size and growth rates were dramatically affected in transgenic mice expressing GH transgenes driven by a metallothionein (MT) enhancer/promoter (Palmiter *et al.*, 1982). From that starting point, similar attempts followed in swine and sheep studies to enhance growth by introduction of various GH gene constructs under control of a number of different regulatory promoters (see Pursel and Rexroad, 1993; Table 1.4). Use of these constructs was intended to allow for tight regulation of individual transgene expression by dietary supplementation. However, although resulting phenotypes included altered fat composition, feed efficiency and rate of gain, and lean:fat body composition, they were accompanied by undesirable side-effects, e.g. joint pathology, skeletal abnormalities, increased metabolic rate, gastric ulcers and infertility (Pursel *et al.*, 1989; Nancarrow *et al.*, 1991). Such problems were attributed to chronic overexpression or aberrant expression of the growth-related transgenes and could be mimicked, in several cases, in normal animals by long-term treatment with elevated doses of GH.

Subsequent efforts to genetically alter growth rates and patterns have included production of transgenic swine and cattle expressing a foreign *c-ski* oncogene, which targets skeletal muscle, and studies of growth in lines of mice and sheep that separately express transgenes encoding growth hormone-releasing factor (GRF) or insulin-like growth factor I (IGF-I). Cumulatively, it has become apparent from these studies that greater knowledge of the biology of muscle growth and development will be required in order to genetically engineer lines of domestic animals with these desired characteristics. However, recent work on IGF-I and GH transgenic pigs reported in this volume (Chapters 10 and 11) indicate that progress is being made.

Other productivity traits that are major targets for genetic engineering include altering the properties or proportions of caseins, lactose or butterfat in milk of transgenic cattle and goats, more efficient wool production, and enhanced resistance to viral and bacterial diseases (including development of 'constitutive immunity' or germline transmission of specific, rearranged antibody genes).

Domestic Animals as Bioreactors

The second general area of interest has been the development of lines of transgenic domestic animals for use as bioreactors. One of the main targets of these so-called 'gene farming' efforts has involved attempts to direct expression of transgenes encoding biologically active human proteins. In such a strategy, the goal is to recover large quantities of functional proteins that have therapeutic value, from serum or from the milk of lactating females. To date, expression of foreign genes encoding α_1-antitrypsin, tissue plasminogen activator, clotting factor IX and protein C were successfully targeted to the mammary glands of goats, sheep, cattle and/or swine (Table 1.5).

Similarly, lines of transgenic swine and mice have been created that produce human haemoglobin or specific circulating immunoglobulins. The ultimate goal of these efforts is to harvest proteins from the serum of transgenic animals for use as important constituents of blood transfusion substitutes, or for use in diagnostic testing.

Commercialization

While transgenic animal technology continues to open new and unexplored agricultural frontiers, it also raises questions concerning regulatory and commercialization issues, as demonstrated by molecular farming efforts. A number of major regulatory and public perception hurdles exists that may affect the time to commercialization of transgenic animals. These include perceptions of genetic engineering motives, ethical considerations including animal welfare issues, and product uniformity and economic production (scale-up) issues. A further issue is the potential environmental impact

Table 1.5. Molecular farming projects: a host of commercial projects are underway using transgenic farm animals as bioreactors to produce important biomedical products (from Pinkert, 1997).

Product	Use	Commercializing firm(s)
α_1-antitrypsin	Hereditary emphysema/cystic fibrosis	PPL
α-glucosidase	Glycogen storage disease	Pharming
Antibodies	Anti-cancer	CellGenesys, Genzyme, Ligand
Antithrombin III	Emboli/thromboses	Genzyme
Collagen	Rheumatoid arthritis	Pharming
CFTR	Ion transport/cystic fibrosis	Genzyme
Factor IX	Blood coagulation/haemophilia	Genzyme, PPL
Fibrinogen	Tissue sealant development	ARC, PPL
Haemoglobin	Blood substitute development	Baxter
Lactoferrin	Infant formula additive	Pharming
Protein C	Blood coagulation	ARC, PPL
Serum albumin	Blood pressure, trauma/burn treatment	Pharming
tPA	Dissolve fibrin clots/heart attacks	Genzyme
Tissues/organs	Engineered for xenotransplantation	Alexion, Baxter, CTI, Novartis

following the 'release' of transgenic animals, particularly fish. These societal issues will exist and will continue to influence the development of value-added animal products through transgenesis until transgenic products and foodstuffs have been proven safe for human use and are accepted by a wide cross-section of society.

Conclusions

The use of transgenic animal models for the study of gene regulation and expression has become commonplace in the biological sciences. However, contrary to the early prospects related to commercial exploitation in agriculture, there are numerous societal challenges regarding potential risks that still lie ahead. The risks at hand can be defined not only by scientific evidence but also in relation to public concern (whether perceived or real). Therefore, the central questions will revolve around the proper safeguards to employ and the development of a coherent and unified regulation of the technology. Will new animal reservoirs of fatal human diseases be created? Will more virulent pathogens be artificially created? What is the environmental impact of the 'release' of genetically engineered animals? But perhaps most importantly, we have to ask the question 'do the advantages of a bioengineered product outweigh potential consequences of its use?'

In spite of the inherent limitations in existing methodologies, transgenic livestock will continue to hold great promise for the agricultural industry. The rate of progress to date has, to a certain degree, been limited by the resources available to the scientific community. The cost of producing transgenic farm animals is high and, thus, it is of no surprise that most efforts are carried out in laboratories receiving large amounts of direct government funding. This is, at least in part, changing as venture capital and industry money is now been put into the development of transgenic livestock to produce pharmaceuticals in transgenic animal bioreactors.

Our role as scientists, consumers and regulators is, in part, to decide at what levels or stages and to what degree the development of agriculturally important transgenic animals must be monitored and regulated to ensure consumer safety and animal well-being, and address societal concerns. A further corollary to this responsibility is to ensure that the consuming public understands the processes to the extent that they can accept government approval of such animals in the food chain.

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2 Development of Genetic Tools for Transgenic Animals

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There is a chronic need to develop transgenic fish for aquaculture and genetically engineered farm animals for various agricultural and medical purposes. To meet this need we have developed new lines of vectors based on transposable elements and border elements for genetically engineering animals in an efficient, cost-effective manner. The principles of two newly developed tools for fish are described. First, a transposable element system, based on the *Tc1/mariner* family of transposons that is active in animals from fish to mammals, is described. Second, the effectiveness of border elements, derived from insects and birds, to insulate transgenes from position effects is presented. Fish are used as a model system and as an example of the type of needs that can be met because these species are especially convenient and inexpensive for use in the development of laboratory procedures.

Introduction

The aquatic resources of the world are being exhausted by over harvesting of finfish and other aquatic organisms. As the world's population grows, its fisheries are being depleted at increasing rates. The USA suffers from a staggering international trade imbalance in fisheries products, about US$3 billion per year, the third largest contributor to its annual imbalance of payments. The US consumption of about 20 kg/person requires the harvest of a total of about 6 Mt per year (Parfit, 1995). To meet this demand, the US fishing fleet is harvesting more fish and depleting the fish stocks required to maintain fish populations, which has led in the last 4 years to a decline in wild fisheries off the coasts of the USA. As a result, in future years we will not be able to produce sufficient quantities of fish for our national needs. The situation will get worse if we continue with a 'fishing as usual' policy.